

This is the **Exhibit D** referred to in the Declaration under
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The Growth Inhibitor of African Green Monkey (BSC-1) Cells Is Transforming Growth Factors $\beta 1$ and $\beta 2$

John M. McPherson,* Steven J. Sawamura, Yasushi Ogawa, Kelly Dineley, Pedro Carrillo, and Karl A. Piez
Celtrix Laboratories, Collagen Corporation, 2500 Faber Place, Palo Alto, California 94303

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ABSTRACT: The growth inhibitory activity in conditioned medium of African green monkey kidney epithelial (BSC-1) cells that has been shown to arise, at least in part, from transforming growth factor $\beta 2$ (TGF- $\beta 2$) [Hanks, S. K., Armour, R., Baldwin, J. H., Maldonado, F., Spiess, J., & Holley, R. W. (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85, 79-82] was tested for growth inhibitory activity prior to and following acidification. Similar to TGF- $\beta 1$ from human platelets, the inhibitory activity from BSC-1 cells demonstrated an 8-10-fold stimulation following acidification, showing that the activity was secreted from the cells in latent form. Conditioned medium from BSC-1 cells was collected, acidified, and fractionated by procedures that separate TGF- $\beta 1$ and -2. Biological activity was assayed by using the BSC-1 cell proliferation assay. Two active proteins with properties similar to known TGF- $\beta 1$ and TGF- $\beta 2$ were identified. Identity was confirmed by using immunological and amino acid sequencing techniques. These results were consistent with Northern blot analysis of total BSC-1 RNA, using cDNA probes for TGF- $\beta 1$ and TGF- $\beta 2$, which demonstrated strong signals for both mRNAs. Metabolic labeling in conjunction with two-dimensional gel electrophoresis revealed that the cells secrete approximately 10% TGF- $\beta 1$ and 90% TGF- $\beta 2$.

The multitude of biological activities attributed to transforming growth factor $\beta 1$ (TGF- $\beta 1$)¹ and TGF- $\beta 2$ is remarkable [for reviews, see Sporn et al. (1987), Massague (1987), and Roberts and Sporn (1988)]. The activity was characterized by Roberts et al. (1980, 1981), who showed that in combination with epidermal growth factor it could promote anchorage-independent growth of NRK fibroblasts, hence, the

name "transforming growth factor". The factor, now designated TGF- $\beta 1$, was shown to be present in many normal tissues

* Author to whom correspondence should be addressed at Integrated Genetics, 1 Mountain Rd., Framingham, MA 01701.

¹ Abbreviations: TGF, transforming growth factor; NRK, normal rat kidney; BSC-1, African green monkey kidney epithelial; GI, growth inhibitor; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; CM, carboxymethyl; RP-HPLC, reverse-phase high-pressure liquid chromatography; TFA, trifluoroacetic acid; ATCC, American type culture collection; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

and was characterized and purified to homogeneity from several tissues (Frolik et al., 1983; Assoian et al., 1983; Roberts et al., 1983; Seyedin et al., 1985). Since then, the factor has been shown to be both a positive and negative growth regulator, to control cell differentiation, to be chemotactic for macrophages and fibroblasts, to enhance production of collagen and fibronectin by fibroblasts in culture, to promote a fibrogenic response in vivo, and to enhance the rate of wound healing in certain animal models. Although this is not a complete list of the biological activities of the protein, it serves to demonstrate its multifunctionality. The mature, active form of the protein is highly conserved, with only one amino acid substitution between mouse and man (Derynck et al., 1985).

A protein, initially designated cartilage inducing factor B, was purified from bovine bone and shown to be related to TGF- β 1 in biologic activity but was only 70% identical in sequence (Seyedin et al., 1985, 1987; Marquardt et al., 1987). This protein has also been purified from pig platelets (Cheifetz et al., 1987) and has been designated TGF- β 2. In most in vitro comparisons, TGF- β 1 and TGF- β 2 have similar activities.

African green monkey kidney epithelial (BSC-1) cells have been known to secrete substances into the culture media that feed-back inhibit their further proliferation (Holley et al., 1978, 1980). These inhibitors include lactic acid, ammonia, and a protein growth inhibitor of M_r 24,000 that was designated GI (Holley et al., 1980, 1983a). These investigators showed that this protein factor inhibited proliferation of not only BSC-1 cells but also the CCL64 mink lung epithelial cell line, the human mammary tumor cell line Hs578T (Holley et al., 1983b), and to a lesser extent rat lung and human breast epithelial cells (Holley et al., 1980). GI did not influence 3T3 cell or human skin fibroblast proliferation. Subsequently, it was demonstrated that GI was related to TGF- β 1 (Tucker et al., 1984). Recently, Hanks et al. (1988) showed, on the basis of the partial amino acid sequence and the nucleotide sequence of cDNA, that the BSC-1 GI, as isolated by them, was TGF- β 2. However, the coincident presence of TGF- β 1 was not ruled out.

The objectives of our experiments were to determine (1) whether TGF- β 1 as well as TGF- β 2 is secreted by BSC-1 cells and (2) whether the active or latent form is secreted. The results presented here provide definitive evidence that the growth inhibitory activity secreted by BSC-1 cells is a combination of TGF- β 1 and TGF- β 2, predominantly in latent form.

EXPERIMENTAL PROCEDURES

Materials. The BSC-1 cells used in these experiments were a kind gift of Dr. Robert Holley (Salk Institute, La Jolla, CA). Dr. Holley also provided a plasmid from which we constructed a cDNA probe for TGF- β 2. The TGF- β 1 cDNA probe used in our experiments was obtained from Dr. Anthony Purchio (Oncogen, Seattle, WA). The anti-TGF- β 1 antibody used in Western blots in this study was provided by our colleague Dr. Larry Ellingsworth (Ellingsworth et al., 1986). Purified TGF- β 1 and TGF- β 2 were prepared from bovine bone as previously described (Seyedin et al., 1985, 1986, 1987). The DL-[4,5- 3 H]leucine used in metabolic labeling studies was purchased from Amersham Corp. (Arlington Heights, IL).

Cell Culture Methods. BSC-1 cells were grown to confluence in 20 24.5 \times 24.5 cm tissue culture plates (NUNC, Irvine Scientific) in DMEM with 0.45% glucose, supplemented with 10% fetal bovine serum, 0.5 μ g/mL biotin, and 1% penicillin/streptomycin as previously described (Holley et al., 1980; Nilsen-Hamilton & Holley, 1983). Conditioned medium was prepared by first washing confluent cell layers twice with

PBS and then introducing approximately 75 mL of culture medium minus serum into each dish and incubating the cells for 24 h at 37 $^{\circ}$ C. Conditioned medium was removed and replaced with DMEM containing 0.2% serum and the other culture additives mentioned above. This process was repeated twice a week for 7 weeks until 18.4 L of conditioned medium was collected. Collected medium was stored frozen at -20 $^{\circ}$ C.

Growth Inhibitor Purification. Conditioned medium was thawed, and urea was added, with stirring, to a final concentration of 6 M, and phenylmethanesulfonyl fluoride was added to a final concentration of 0.2 mM. The medium was concentrated to 1.5 L and diafiltered at 4 $^{\circ}$ C against a buffer containing 50 mM sodium acetate, pH 4.6, 10 mM NaCl, and 6 M urea (buffer A) using a cross-flow filtration unit (Amicon Model S10Y10, 10 ft² YM 10 membrane). The retentate was clarified by centrifugation at 13700g for 30 min at 4 $^{\circ}$ C. The clarified retentate was loaded on a CM-cellulose column (2.5 \times 37 cm) (Whatman CM-52) equilibrated in buffer A at room temperature, and the column was developed with a 800-mL linear gradient between 10 and 400 mM NaCl in buffer A. Fractions containing growth inhibitory activity for BSC-1 cells were pooled and loaded on a Vydac C-18 RP-HPLC column (4.6 \times 250 mm, 5- μ m particle size) equilibrated in 0.1% TFA. The column was washed in 0.1% TFA and subsequently in 32% acetonitrile/0.1% TFA. The column was developed with a linear gradient between 32 and 62% acetonitrile. Column fractions were analyzed by in vitro assay, SDS-PAGE (Laemmli, 1970; Studier, 1978; Morissey, 1981), and immunochemically using immunoblots (Ellingsworth, 1986). Selected fractions were diluted with an equal volume of 0.1% TFA and rechromatographed on RP-HPLC under the same conditions.

Cell Culture Assays. Assays to test the inhibition of BSC-1 cell proliferation by conditioned medium and various column fractions were performed in the following manner. Cells were detached from confluent monolayers with trypsin and suspended in high-glucose DMEM supplemented with nonessential amino acids, sodium pyruvate, biotin (0.4 μ g/mL), and 10% fetal bovine serum at approximately 40,000 cells/mL. Assays were performed in 96-well microtiter plates (Costar) with approximately 2000 cells initially introduced per well. Cells were allowed to attach for at least 30 min, and then samples to be tested were introduced into the wells and incubated for 48–72 h at 37 $^{\circ}$ C. Following incubation, 1 μ Ci of [3 H]thymidine in 50 μ L of culture medium was added to each well, and the cells were incubated an additional 18–24 h. Medium was then removed from the cells; the wells were washed twice with phosphate-buffered saline and once with methanol. The cell layers were allowed to air-dry and were dissolved in 200 μ L of 1 M NaOH by incubation for 2 h at room temperature. Aliquots were removed, and the radioactivity was quantitated by liquid scintillation counting in Scinti Verse II (Fisher Scientific; Fair Lawn, NJ) using a Beckman LS 5801 scintillation counter (Beckman Instruments; Palo Alto, CA). Data acquisition, statistical analyses, and conversion of cpm to dpm were performed with a Hewlett-Packard 9816 microcomputer (Hewlett-Packard; Palo Alto, CA) that was interfaced with the scintillation counter, using a network that has been previously described (Kulis, 1984; Condell, 1986). Analysis of various fractions for their ability to promote anchorage-independent growth of nontransformed cells in soft agar was performed according to the method of Roberts et al. (1984). NRK fibroblasts, clone 49 F, were obtained from frozen cultures (ATCC, CRL 1570). Epidermal growth factor (Bethesda Research Laboratories, Gaithersburg,

MD) was present at 5 ng/mL in the cultures.

Metabolic Labeling of BSC-1 Cell Conditioned Media. BSC-1 cells were cultured as described above in 150-mm tissue culture plates. At 50–80% confluency, the culture medium was removed, and the cell layers were washed twice with 10 mL of phosphate-buffered saline. The cells were metabolically labeled by adding culture media (minus serum) containing DL-[4,5-³H]leucine at a concentration of 50 μ Ci/mL, followed by incubation at 37 °C for 36 h. The culture media from six plates (approximately 20 mL/plate) were harvested and combined with 5 mL of phosphate-buffered saline washes from each plate. The radiolabeled conditioned medium was dialyzed against 4 L of 0.1% acetic acid at 4 °C with three buffer changes. The conditioned medium was lyophilized, redissolved in 10 mL of 1% acetic acid, and relyophilized. The sample was then dissolved in a small volume of 1 M acetic acid, 6 M urea, and 1% methyl green and subjected to two-dimensional gel electrophoresis as previously described (Ellingsworth, 1986). The gel was prepared for fluorescent autoradiography by treatment with Enhance (Dupont/NEN; Boston, MA). The gel was then dried on filter paper and exposed to X-ray film. Similar conditioned media samples were spiked with purified TGF- β 1 and TGF- β 2 and subjected to two-dimensional gel electrophoresis, and the gels were silver stained (Morrissey, 1981) to identify the electrophoretic positions of TGF- β 1 and TGF- β 2.

Northern Blot Analysis. Total RNA was extracted from BSC-1 cells (8×10^8 cells in 1 mL) with guanidinium and cesium chloride as described by Maniatis et al. (1982). The RNA (4 μ g) was fractionated on a 1% agarose-formaldehyde gel, transferred to a nylon membrane (Schleicher & Shuell Inc.; Keene, NH) and then hybridized with a ³²P-labeled cDNA probe. Hybridization with the TGF- β 1 and TGF- β 2 probes was performed at 42 °C in 50% formamide, 20 \times buffer B (3 M sodium chloride/3 M sodium citrate, pH 7), 2 M Tris (pH 7.4), 100 \times Denhart's solution, dextran sulfate, and 2 mg/mL salmon sperm DNA. Filters were washed at 55 °C for 20 min in 2 \times buffer B and 0.1% SDS, followed by two washes in 0.1 \times buffer B and 0.1% SDS for 10 min each at room temperature. The filters were dried and exposed for 10 h to X-ray film.

RESULTS

Conditioned, serum-free medium from BSC-1 cells was tested for its ability to inhibit BSC-1 cell proliferation prior to and following acidification. Assaying the medium at several different dilutions revealed that acidification significantly increased inhibitory activity and resulted in an 8–10-fold stimulation in inhibitory activity when assayed at the highest dilution (Figure 1). The growth inhibitory activity in unfractionated medium has been attributed to several components (Holley et al., 1978, 1980), but the predominant one after acidification is apparently TGF- β 2 (Tucker et al., 1984; Hanks et al., 1988). TGF- β 1 purified from platelets, and most other sources, exists in latent form and requires acidification or treatment with denaturing agents to express full activity (Lawrence et al., 1984; Pircher et al., 1984). The results of our experiments suggest that the majority of growth inhibitory activity secreted by BSC-1 cells also exists in latent form.

Approximately 18 L of conditioned, serum-free BSC-1 media was concentrated to 1.5 L by cross-flow filtration in the presence of 6 M urea as described under Experimental Procedures. This level of urea is sufficient to completely activate latent TGF- β 1 or TGF- β 2 (Y. Ogawa, unpublished observation). The concentrate was applied to a CM-cellulose cation-exchange column that was developed with a linear sodium

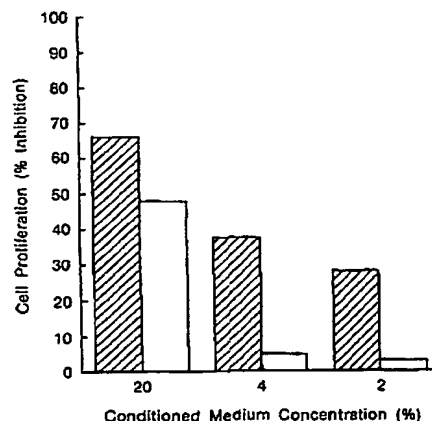


FIGURE 1: Effect of acidification on the growth inhibitory activity of BSC-1 cell conditioned media. BSC-1 media were collected following 24 h of cell culture, and a portion was acidified to pH 2 and reneutralized as described under Experimental Procedures. Increasing dilutions of acidified (hatched bars) and nonacidified (open bars) media were made in fresh media containing 10% fetal bovine serum. The various diluted samples were tested for their ability to inhibit BSC-1 cell proliferation as measured by reduced [³H]thymidine incorporation. Values provided are the average of triplicate determinations.

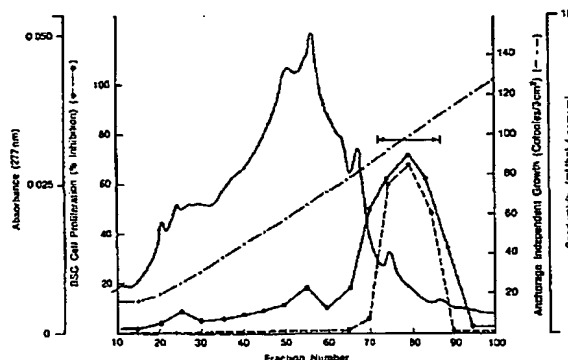


FIGURE 2: Cation-exchange chromatography of BSC-1 cell conditioned media. Conditioned medium from BSC-1 cells was harvested, concentrated to 1.5 L, and subjected to CM-cellulose chromatography as described under Experimental Procedures. The column was developed with a linear salt gradient between 0.01 and 0.4 M NaCl in a buffer containing 0.05 M sodium acetate (pH 4.6) and 6 M urea. Absorbance at 277 nm (—), inhibition of BSC-1 cell proliferation (●—●), and promotion of NRK cell anchorage-independent growth (○—○) were monitored in the column fractions. Fractions 70–88, identified by arrows, were pooled for further purification.

chloride gradient. Biological activity of the fractions was assayed by using the BSC-1 growth inhibitory activity assay in conjunction with the NRK fibroblast anchorage-independent proliferation assay (Roberts et al., 1984). Both of these activities coeluted in a broad peak between conductivities of 9 and 11 m Ω ⁻¹ (Figure 2).

Fractions containing biological activity of interest were pooled and applied to a C-18 RP-HPLC column. The column was developed with a linear acetonitrile gradient. The various fractions from this column were assayed for their ability to inhibit BSC-1 cell proliferation. Two peaks of growth inhibitory activity were detected (Figure 3). The retention time of the first peak corresponded to that of TGF- β 1 while that of the more prominent second peak corresponded to TGF- β 2 as previously observed for the factors from bovine bone (Seyedin et al., 1985). SDS-PAGE demonstrated that these growth inhibitory fractions contained protein bands that co-

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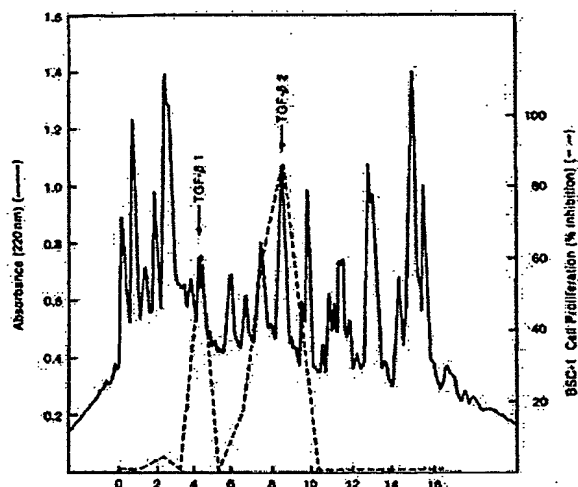


FIGURE 3: RP-HPLC of BSC-1 cell conditioned media. Pooled fractions from the CM-cellulose column were applied to a C-18 RP-HPLC column which was equilibrated to 0.1% TFA and developed with an acetonitrile gradient as described under Experimental Procedures. Growth inhibitory activity was assayed by inhibition of BSC-1 cell proliferation as measured by [3 H]thymidine incorporation. This assay provides a linear dose response to only about 40% inhibition, and thus the relative amounts of TGF- β 1 and TGF- β 2 cannot be determined from this profile. Fractions corresponding to retention times of TGF- β 1 and TGF- β 2 from bovine bone are identified.

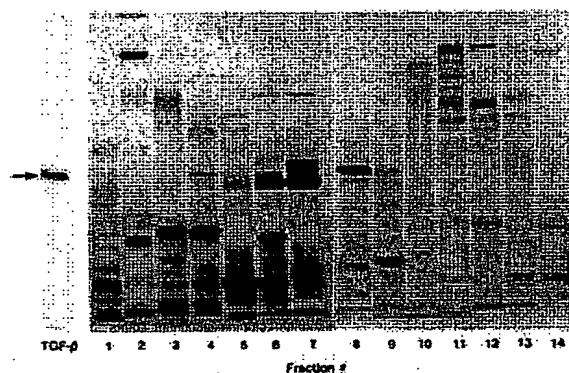


FIGURE 4: SDS-PAGE of RP-HPLC fractions. Aliquots of individual fractions were subjected to SDS-PAGE (Laemmli, 1970; Studier, 1978) on a 15% gel, and proteins were identified by using a sensitive silver-staining technique (Morrissey, 1981). The mobility of purified TGF- β 1 from bovine bone in this system is illustrated in the left lane (see arrow); TGF- β 2 has the same mobility. Proteins of similar mobility can be seen in fractions 4 and 7-9.

migrated with purified TGF- β 1 and TGF- β 2 from bovine bone under nonreducing conditions (Figure 4).

Western blot analysis was performed on aliquots of the HPLC fractions using an antibody that was specific for TGF- β 1 (Ellingsworth et al., 1986). A strong signal was detected in fraction 4 (peak 1) whose mobility corresponded to that of TGF- β 1 (Figure 5). A weak signal was noted in fraction 9 with a mobility corresponding to reduced TGF- β 1. Fraction 8 exhibited no significant signal although it contained most of the inhibitory activity in the effluent from the RP-HPLC column (Figure 3).

Fractions containing the second peak of activity from the RP-HPLC column (Figure 3) were pooled and rechromatographed on the same column under identical conditions. This procedure provided a growth inhibitory fraction whose protein

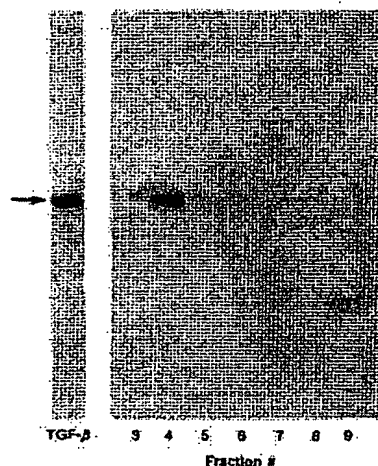


FIGURE 5: Fluorescence autoradiogram from immunoblot analysis of HPLC fractions developed with an antibody directed against TGF- β 1. HPLC fractions were resolved by SDS-PAGE as described. The proteins were transferred to nitrocellulose paper, and the blot was developed with an antibody with high specificity to TGF- β 1 (Ellingsworth, 1986). Antibody binding was detected by using an [125 I]-labeled second antibody followed by autoradiography. The mobility and reactivity of TGF- β 1 in this system are illustrated in the left lane (see arrow).

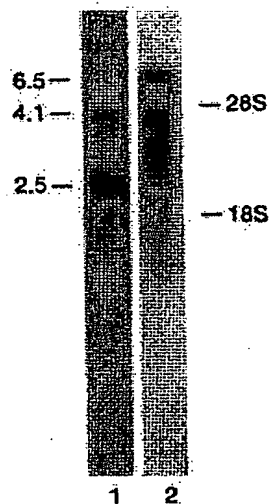


FIGURE 6: Northern blot analysis of total RNA from BSC-1 cells using probes directed against TGF- β 1 and TGF- β 2. Total RNA was extracted from BSC-1 cells and subjected to Northern blot analysis as described under Experimental Procedures. The electrophoretically resolved RNA was hybridized with cDNA probes to TGF- β 1 (lane 1) and TGF- β 2 (lane 2). The migratory positions for the 2.5- and 4.1-kb mRNA of TGF- β 1 (Sharples et al., 1987) and the 4.1- and 6.5-kb mRNA of TGF- β 2 (Madisen et al., 1988), respectively, are identified.

purity was judged to be greater than 95% based on SDS-PAGE and whose mobility was identical with that of TGF- β 2 under reducing and nonreducing conditions (data not shown). Sequence analysis of the first 12 amino acids of the protein showed them to be identical with those of bovine TGF- β 2 (Seyedin et al., 1987). Independent confirmation that these cells could produce both TGF- β 1 and TGF- β 2 was obtained by Northern blot analysis of total RNA from these cells. These experiments showed that BSC-1 cells contained mRNA transcripts for both TGF- β 1 and TGF- β 2 (Figure 6).



FIGURE 7: Fluorescence autoradiogram of two-dimensional gel electrophoresis of metabolically radiolabeled BSC-1 cell conditioned medium. BSC-1 cells were metabolically labeled for 36 h at sub-confluency with [3 H]leucine as described under Experimental Procedures. The radiolabeled conditioned medium was collected, concentrated, and subjected to two-dimensional gel electrophoresis and fluorescence autoradiography as previously described (Ellingsworth et al., 1986). The positions of authentic TGF- β 1 and TGF- β 2 in the two-dimensional gel are identified.

Metabolic labeling studies were performed to provide final confirmation that BSC-1 cells synthesize both TGF- β 1 and TGF- β 2 and to quantitate the relative amounts of the two factors. Conditioned, serum-free medium from cells radiolabeled with [3 H]leucine was concentrated and subjected to two-dimensional gel electrophoresis as described under Experimental Procedures. Fluorescence autoradiography of the gel revealed two spots whose mobility corresponded to purified TGF- β 1 and TGF- β 2 from bovine bone (Figure 7). Similar samples were spiked with purified, nonradiolabeled TGF- β 1 and TGF- β 2 from bovine bone, subjected to two-dimensional gel electrophoresis, and silver stained, and the radioactivity in the migratory positions of TGF- β 1 and TGF- β 2 was quantitated by elution and scintillation counting. This experiment indicated that the cells produced approximately 90% TGF- β 2 (9338 dpm) and 10% TGF- β 1 (874 dpm).

DISCUSSION

TGF- β 1 obtained from human platelets, and presumably most other tissue sources, exists in a latent form which in vitro requires acidification or treatment with denaturing agents for full activity (Lawrence et al., 1984; Pircher et al., 1984). TGF- β 1 synthesized and secreted by most cells in culture also exists in latent form (Roberts et al., 1986; Assoian et al., 1987; Robey et al., 1987; Wakefield et al., 1987). We show here that the majority of inhibitory activity secreted by BSC-1 cells also exists in latent form. Since the inhibitory activity secreted by these cells is composed of several components including lactic acid and ammonia as well as TGF- β 1 and TGF- β 2, it is not possible to determine what component is responsible for activity in nonacidified medium. However, on the basis of dilution experiments, we conservatively estimate that >80% of the activity exists in latent form. This may be compared to platelets in which 98–99% of the TGF- β 1 exists in latent form (Pircher et al., 1986). Since TGF- β 2 represents most of the activity in acidified medium, it must be present largely in latent form in nonacidified medium.

The biochemical nature of the latent forms of TGF- β 1 and TGF- β 2 as well as the method of activation in vivo is incompletely understood. α_2 -Macroglobulin reportedly forms a

complex with platelet-derived TGF- β 1 which is acid sensitive (O'Connor-McCourt & Wakefield, 1987). Plasmin can partially activate latent TGF- β 1 from platelets and is therefore believed to be a mechanism of factor activation in vivo (Keskitalo et al., 1987). The significance of the latent form is unclear, but latency presumably represents an important control mechanism in the expression of activity of this factor.

The fact that the latent form is produced in vitro suggests that putative binding proteins involved in controlling its activity are also synthesized by the cells. Since the conditioned medium was collected under serum-free conditions, it is unlikely that inactivation occurred outside of the cell by binding to some serum factor such as α_2 -macroglobulin.

The data we have presented indicate that although BSC-1 cells produce predominantly TGF- β 2, they do produce significant levels of TGF- β 1, representing about 10% of the total activity obtained from the conditioned medium. The possibility existed that the TGF- β 1 we purified was actually derived from the bovine serum in which the cells were cultured prior to harvesting of conditioned, serum-free media. However, Northern blot analysis showed that these cells produce mRNAs for both forms of the factor consistent with the amounts found in the medium. Final evidence for synthesis of both factors was provided by metabolic incorporation of label into both. BSC-40 cells, a subline of BSC-1, have also been shown to contain mRNA for TGF- β 1 (Sharples et al., 1987). Furthermore, metabolic labeling studies, in conjunction with two-dimensional gel electrophoresis, provided evidence that BSC-1 cells do indeed synthesize low levels of TGF- β 1, in amounts consistent with what was found.

As noted earlier, the cells we utilized in these experiments were obtained from Dr. Robert Holley (Salk Institute, La Jolla, CA). BSC-1 cells obtained from the ATCC were very different in morphology and proliferative activity from those obtained from Dr. Holley. Metabolic labeling studies with ATCC cells revealed a significantly different biosynthetic profile, although they also produced predominantly TGF- β 2 (data not shown). Our results are not inconsistent with those of Hanks et al. (1988), who found that BSC-1 cells made TGF- β 2. Their RP-HPLC pattern (see their Figure 1) showed a minor peak of activity, in addition to TGF- β 2, which can now be ascribed to TGF- β 1.

The driving force for BSC-1 cells to produce both TGF- β 1 and TGF- β 2 is unclear at this time. We have observed that both forms inhibit BSC-1 cell proliferation to a similar degree (Y. Ogawa, unpublished observations). Pig platelets have been reported to contain both TGF- β 1 and TGF- β 2, and thus pig megakaryocytes must also actively produce both forms of the factor. Although most of the in vitro biologic activities of TGF- β 1 and TGF- β 2 are similar, some differences have been reported (Ohta et al., 1987; Rosa et al., 1988). It is likely that separately regulated induction, synthesis, and activation as well as different biological activities of TGF- β 1 and TGF- β 2 will be found to play a role in their function.

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